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Involvement of Cysteine Residues in the Function and Inactivation of PCP Hydroxylase

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Involvement of Cysteine Residues in the Function and Inactivation of PCP Hydroxylase

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Abstract

The alteration of a microbial environment by introduction of a new xenobiotic chemical leads to the evolution of new metabolic pathways so that the microbes can detoxify them and utilize them as carbon sources. Pentachlorophenol (PCP) is a toxic pollutant that was introduced into the environment in the 1930's. The Gram-negative bacterium *Sphingobium chlorophenolicum* strain [L-1 ATCC 53874] was isolated from soil contaminated with PCP and has been observed to be able to degrade PCP.

The first enzyme in the PCP degradation pathway in this bacterium, PCP hydroxylase, catalyzes the rate-limiting step in the pathway. Along with its poor efficiency, this enzyme is inactivated by its product, tetrachlorobenzoquinone (TCBQ). It is suggested that this inactivation occurs as a result of the covalent attachment of TCBQ to cysteine residues on the enzyme.

I have found that changing cysteine residues 302 and 537 to serines does not significantly affect the activity of the enzyme, and changing cysteine residue 537 to serine slightly reduced the degree of inactivation by TCBQ. The C537S mutant version of the enzyme was about three-fold inactivated, while the wild type version was about four-fold inactivated.

Table of Contents

Acknowledgements

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Chapter 1: Background

The introduction of new toxic xenobiotic compounds leads to the evolution of new metabolic pathways that degrade these compounds to detoxify them and potentially allow the organism to utilize these compounds as carbon sources. Pentachlorophenol (PCP) has been used as a pesticide and wood preservative since the 1930's. Since PCP is a polychlorinated aromatic compound, PCP is highly resistant to degradation. Thus, PCP has been accumulating in the environment since the start of the use of PCP by humans and has not been efficiently removed from the environment by microorganisms. PCP is now recognized as a major environmental pollutant by the EPA and is highly toxic in that PCP uncouples electron transport and oxidative phosphorylation and perturbs membrane properties (Copley, 2000, Tiirola, et. al., 2002).

The Gram-negative bacterium *Sphingobium chlorophenolicum* strain [L-1 ATCC 53874], having been isolated from soil contaminated with PCP, is one of the few bacteria that can degrade PCP. Although mono- and dichlorophenols are naturally produced by fungi and insects, PCP has not been naturally produced, so the recent introduction of PCP into the environment by humans is the first exposure microbes have had to PCP. Having only been exposed to PCP for a short time by evolutionary standards, the bacterium has not had sufficient time to develop an efficient PCP degradation pathway, so this pathway is very imperfect (Crawford, et. al., 2007, Dai, et. al., 2003, Tiirola, et. al., 2002). The bacterium's ability to degrade PCP is hindered at higher concentrations of PCP, and the bacterium does not survive concentrations of 60 mg/L of PCP or higher (Tiirola, et. al., 2002, Yang, et. al., 2006). Engineering the pathway to be more efficient and utilizing the pathway would be an ideal way to clear PCP from the environment.

The first enzyme in the *S. chlorophenolicum* PCP degradation pathway is the flavoprotein PCP hydroxylase (PcpB), which catalyzes the hydroxylation of PCP para to the hydroxyl group, producing tetrachlorobenzoquinone (TCBQ). This step requires NADPH and O_2. PcpD catalyzes the reduction of TCBQ to tetrachlorohydroquinone (TCHQ). This step also requires NADPH. TCHQ then undergoes two dechlorination steps, catalyzed by PcpC. The resulting aromatic ring is cleaved by PcpA. The cleaved

product undergoes multiple other steps to be completely oxidized to form carbon dioxide and hydrochloric acid (Figure 1).

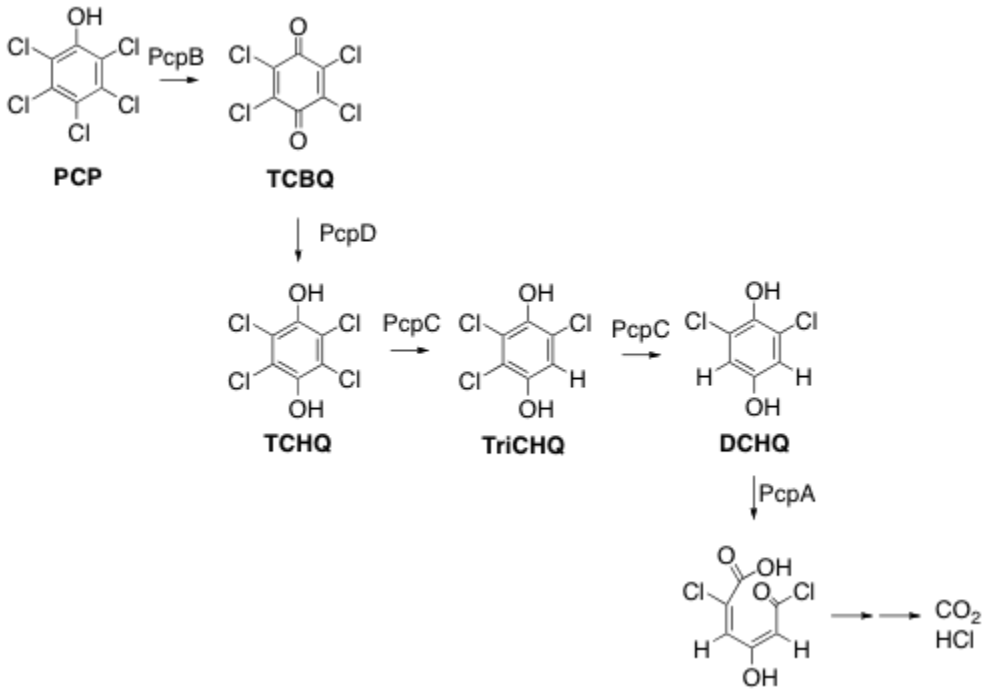


Figure 1. PCP degradation pathway in *S. chlorophenolicum.* PcpB, PCP hydroxylase; PcpD, TCBQ reductase; PcpC, TCHQ dehalogenase; PcpA, 2,6-dichlorohydroquinone dioxygenase.

PcpB requires a flavin cofactor and NADPH and O_2 as substrates. The flavin is reduced by NADPH. O_2 is then added to the reduced flavin anion, forming a peroxy-flavin intermediate. PCP de-aromatizes, and the activated carbon para to the hydroxyl group serves as a nucleophile to obtain the hydroxyl group from the peroxy-flavin. Since chloride is a better leaving group than hydroxide, the chloride leaves. The proton of the newly added hydroxyl group is extracted by the flavin, and the ring forms tetrachlorobenzoquinone. After the dissociation of the TCBQ, the C4a-hydroxyflavin eliminates water to regenerate the oxidized flavin ready for the next catalytic cycle (Figure 2).

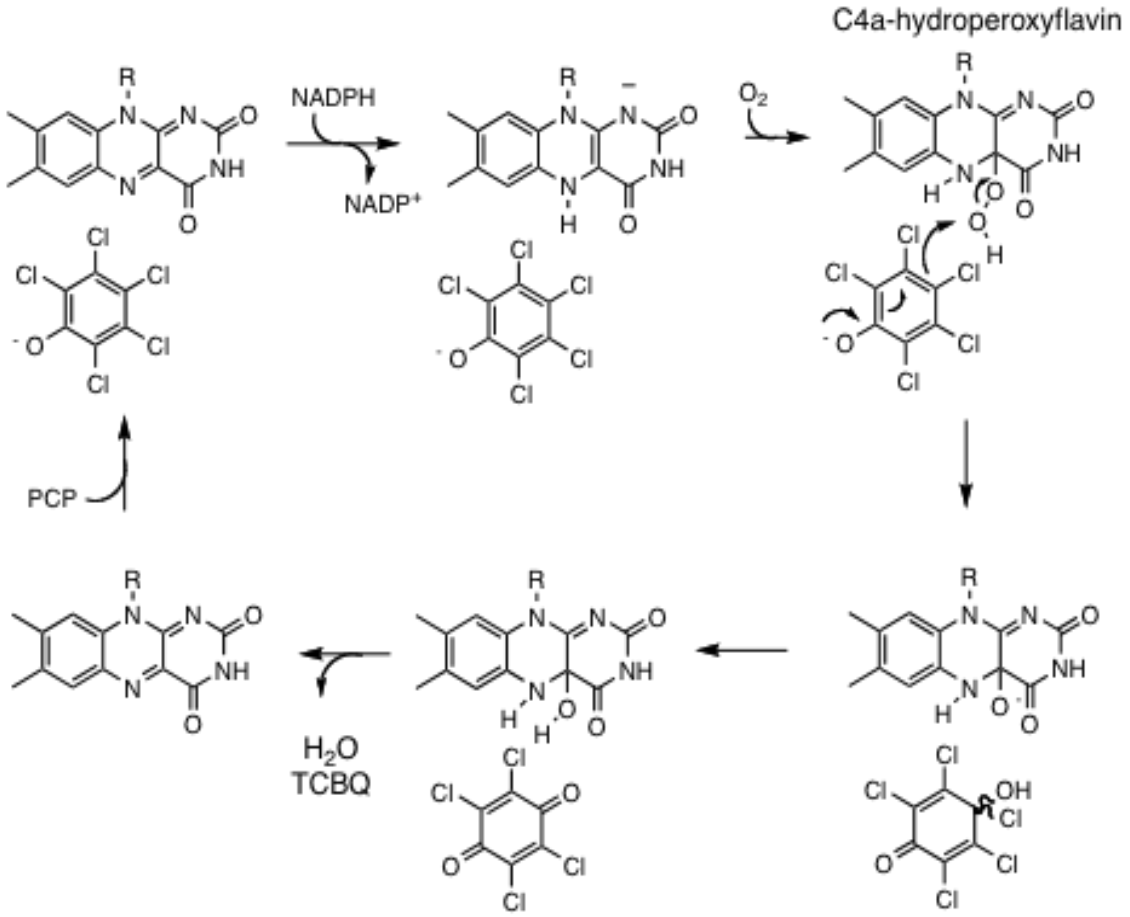


Figure 2. Mechanism of PCP hydroxylase.

The step catalyzed by PcpB is the rate-limiting step in the pathway because PcpB is quite inefficient. This enzyme has probably not yet evolved to its optimal performance, which may be partly due its broad specificity. Many other polyhalogenated phenols besides PCP are also substrates for this enzyme (Copley, 2000, Tiirola, et. al., 2002).

Another negative attribute of this enzyme is that the enzyme allows for futile cycling of NADPH. In this case, NADPH is wasted as the peroxy-flavin dissociates into flavin and hydrogen peroxide. Also, because of the broad specificity of the enzyme, TCHQ can be a substrate for the enzyme. The mechanism is the same as that for PCP, but the TCHQ forms an intermediate with two hydroxyl groups in the position para to the original hydroxyl group on PCP. One of the two hydroxyl groups leaves, forming TCBQ. Since TCHQ is a downstream metabolite of TCBQ in the PCP degradation pathway in *S.*

chlorophenolicum, the conversion of TCHQ back to TCBQ by PcpB makes the progression of PCP and its metabolites through the pathway less efficient by unnecessarily undergoing the rate-limiting step in the pathway and going backwards in the pathway. Also, the use of TCHQ as a substrate by PcpB wastes NADPH by unnecessarily oxidizing NADPH in the conversion of TCHQ to TCBQ and then having to re-reduce TCBQ to TCHQ using NADPH via catalysis by PcpD. However, the ability of PcpB to use TCHQ as a substrate is not proven.

Another undesirable feature is that the enzyme is permanently inactivated by its own product, TCBQ. When the enzyme is inactivated, the number of free cysteine residues in the enzyme decreases, suggesting that the TCBQ inactivates the enzyme by interacting with the cysteine residues. TCBQ may inactivate the enzyme by covalently attaching to the cysteine residues in the enzyme (Figure 3). However, this attachment to cysteine residues is not yet proven. It may also be the case that TCBQ is involved in the oxidation of cysteine residues that must be reduced in order for the enzyme to perform catalysis. TCBQ may catalyze the oxidation of the cysteine residues or may itself react with other compounds in solution, which may lead to the oxidation of the cysteine residues. It seems that the covalent attachment of TCBQ to the cysteine residues is more likely because the aromaticity of TCBQ makes TCBQ a good electrophilic target for a nucleophilic thiol.

It has been observed that many aromatic hydroxylases using this type of mechanism with flavin are inhibited by monovalent anions, such as chloride and azide. Because chloride ions are generated in the conversion of PCP to TCBQ, it may be possible that the accumulation of chloride ions in solution accounts for the inhibition of PcpB as the reaction proceeds. However, it is unlikely that inhibition by chloride ions is the cause of the inactivation of PcpB. From observations of other aromatic hydroxylases using flavin, monovalent anions appear to perturb the C4a-hydroxyflavin intermediate and inhibit the elimination of water from this intermediate, preventing the regeneration of oxidized flavin for the next catalytic cycle (Detmer and Massey, 1984; Maeda-Yorita and Massey, 1993). Because these enzymes manage to proceed to the formation of the C4a-hydroxyflavin intermediate at all, it appears that monovalent anions do not exhibit inhibitory effects early in the catalytic cycle. The inhibitory effect only

occurs upon the formation of the C4a-hydroxyflavin intermediate, which requires NADPH. However, the inactivation of PcpB has been observed due to treatment of the enzyme with TCBQ alone. Even if chloride ions were to form in solution, perhaps due to the reaction of TCBQ with water, chloride ions are not responsible for the observed inactivation of PcpB by TCBQ because NADPH is not in solution during incubation with TCBQ. Any chloride ions that may possibly form during the incubation with TCBQ are removed before the enzyme can Also, Detmer and Massey (1984) report that inhibition of phenol hydroxlase by chloride ions is reversible, whereas inactivation of PcpB by TCBQ is permanent.

Attachment of TCBQ to cysteine resiues near the active site might interfere with the ability of PCP or NADPH to bind to the active site or might interfere with the conformational changes involved in the catalysis. If the enzyme is inactivated by the attachment of TCBQ to a cysteine residue close to the active site, then this would suggest that TCBQ may competitively inhibit PCP from entering the active site or may interfere with conformational changes required for catalysis. If the enzyme gets inactivated by the attachment of TCBQ to a cysteine residue far from the active site, then this would suggest that the TCBQ affects the conformation of the enzyme or its ability to undergo the necessary conformational changes during catalysis. The structure of PcpB is not yet known, but based on the structure of phenol hydroxylase from *Trichosporon cutaneum*, which is 20% identical to PcpB, the cysteine residues at positions 56, 182, and 302 are predicted to be close to the active site (Figure 4).

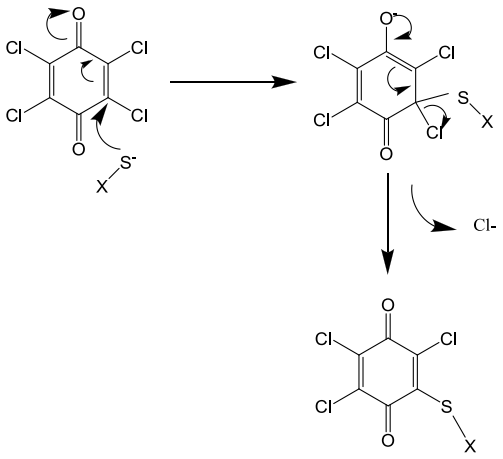


Figure 3. Mechanism of reaction between TCBQ and thiols.

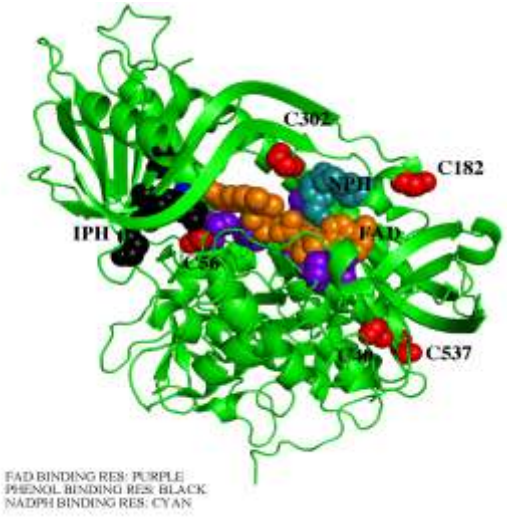


Figure 4. Structure of one subunit of tetrameric phenol hydroxylase from *Trichosporon cutaneum* (PDBID: 1PN0), which is homologous to PCP hydroxylase. Residues that contact the FAD, phenol, and NADPH are shown in purple, black, and cyan, respectively. Orange spheres represent FAD. Red spheres correspond to cysteine residues in PCP hydroxylase. (Courtesy of Dr. Oyeyemi, University of Colorado).

This project aims to determine which cysteine residue(s) in the enzyme is/are involved in the inactivation of the enzyme by TCBQ. In mutant versions of the enzyme, cysteine residues were changed to serine residues. Hopefully, at least one such mutant version of the enzyme will not get inactivated by TCBQ and still be able to perform its catalytic function. If a cysteine residue is required for function and is involved in the inactivation, then preventing the inactivation by removing this cysteine residue while maintaining an enzyme that is still functional is not possible. Upon establishing such a mutant version of the enzyme, we will have more insight into the mechanism of inactivation of the enzyme by TCBQ. The position of the particular cysteine residue that is mutated will provide insight into the mechanism of inactivation. Also, the fact that this mutant version of the enzyme will still be capable of its catalytic function would provide some insight into the catalytic mechanism of the enzyme in that the particular cysteine residue that is mutated is not required for catalysis. This is part of a larger goal to engineer this PCP degradation pathway to be able to clear PCP from the environment more efficiently.

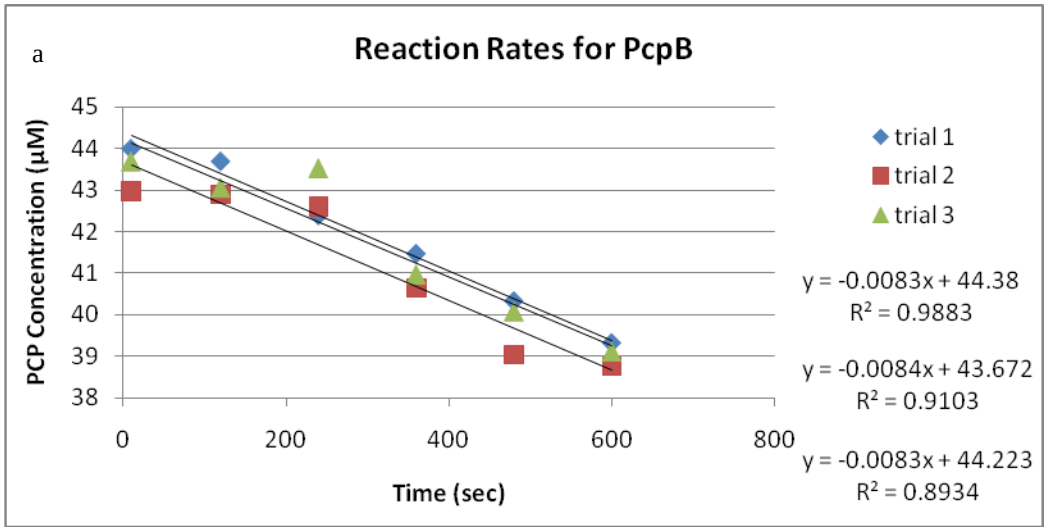
Chapter 2: Effect of Changing Cysteines to Serines on Enzyme Activity

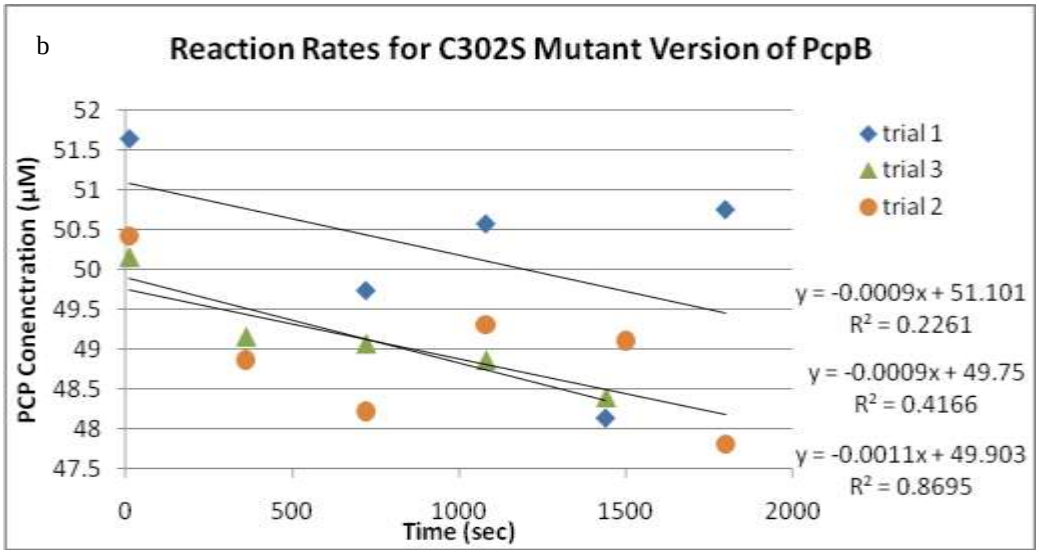
Wild type, C302S and C537S mutant versions of PcpB were assayed for activity by monitoring PCP disappearance by HPLC. Examples of the data are presented in Figure 5. The enzymes were assumed to be saturated with PCP at the concentrations tested because the K_M for PCP for PcpB is about 2 µM, so the K_M for the mutant versions of the enzyme are assumed to be the same. Under this assumption, the PCP concentrations tested were well above the minimal concentration needed to saturate the enzymes. Assays were done only for enzyme saturated with PCP because the peaks at A_{305} for lower concentrations of PCP were too indistinguishable from random noise in the absorption. Values for k_{cat} are given in Table 1 (n=11 for Wt, n=7 for C302S mutant version, and n=3 for C537S mutant version). Because the C302S and C537S mutant versions of PcpB are still functional, none of these cysteine residues is necessary for catalysis.

Table 1. Flavin loading and k_{cat} for wild type and mutant versions of PcpB

PcpB	Flavin Loading	k_{cat} (sec^{-1})
Wt	32%	0.017 ± 0.005
C302S	68%	0.017 ± 0.017
C537S	38%	0.02 ± 0.013

a) Values for k_{cat} are calculated based upon the concentration of flavin.





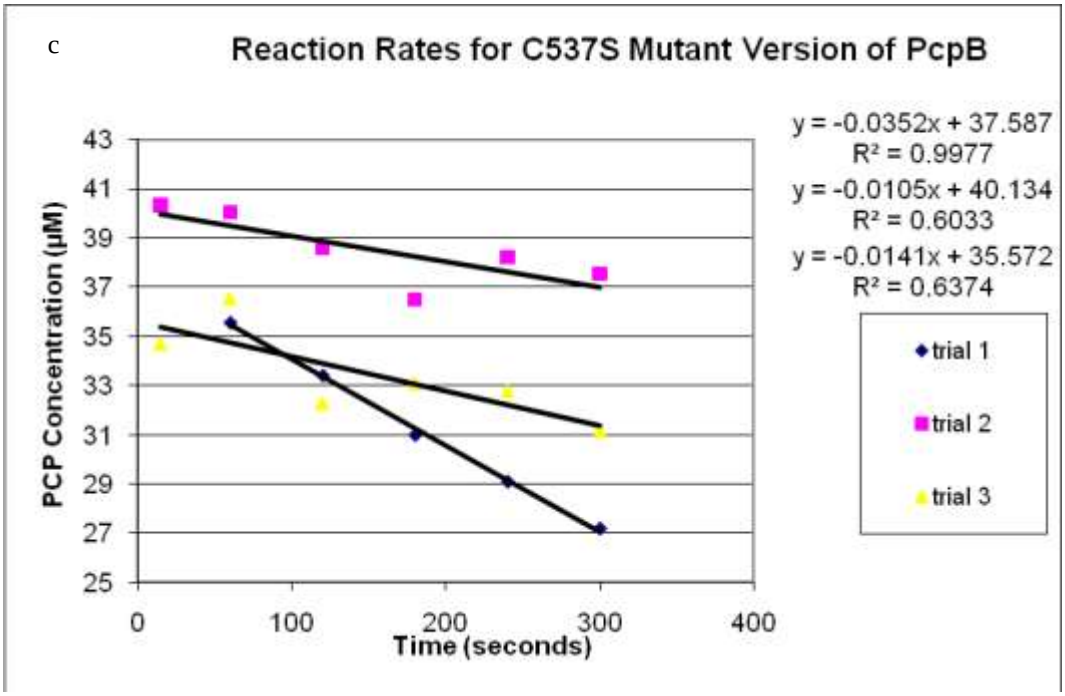


Figure 5. Reaction rates for a) PcpB, b) C302S mutant version of PcpB, and c) C537S mutant version of PcpB. The equations for best-fit lines are in order by trial.

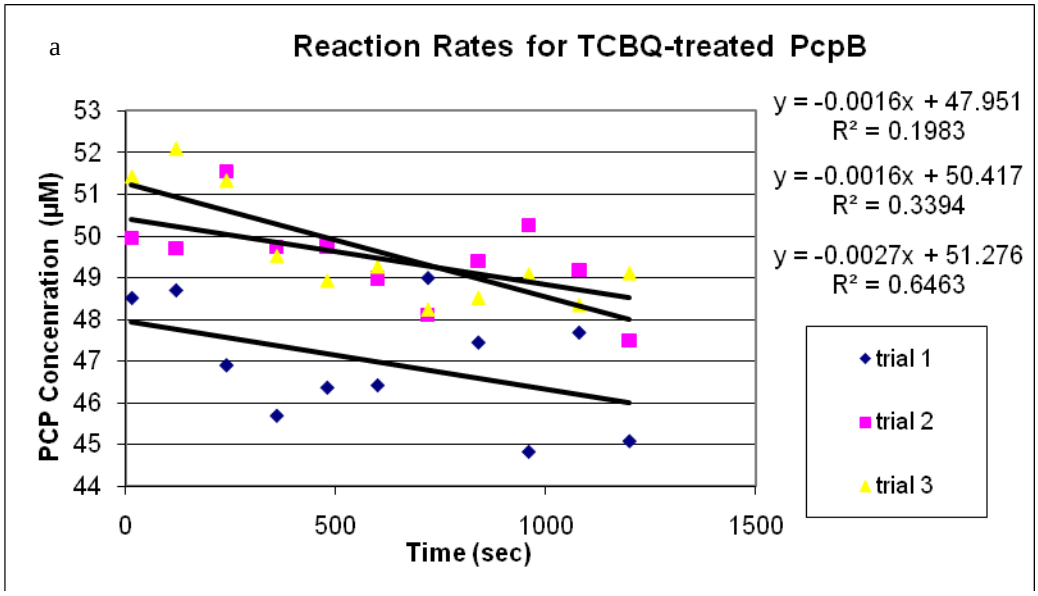
Chapter 3: Inactivation of Enzymes by TCBQ

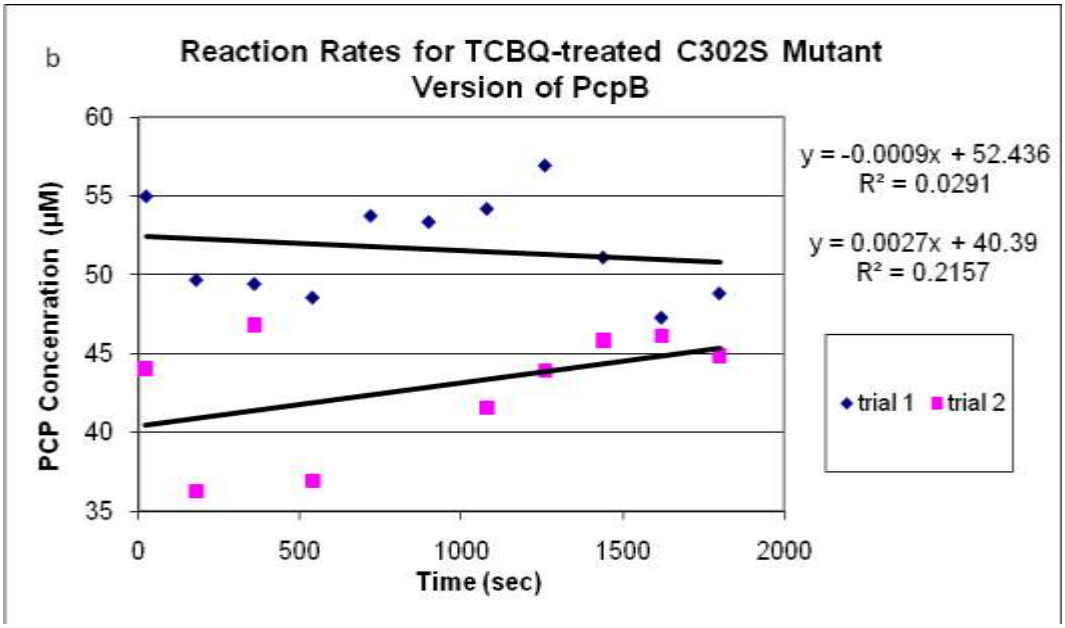
PcpB appears to be inactivated after treatment with 500 µM TCBQ for 60 minutes. This was confirmed by monitoring PCP consumption by HPLC (n=11 for untreated PcpB, n=9 for TCBQ-treated PcpB). Examples of the data are presented in Figure 6. When monitoring PCP consumption by HPLC, reduced reaction rates were observed for TCBQ-treated enzyme (Table 2).

Table 2. $k_{cat,\ PCP}$ for wild type and mutant versions of PcpB treated with 500 µM TCBQ for 60 minutes and enzymes not treated with TCBQ

PcpB	$k_{cat,\ PCP}$ for PcpB Treated with TCBQ (sec^{-1})	$k_{cat,\ PCP}$ for PcpB Not Treated with TCBQ (sec^{-1})
Wt expressed in Tuner (DE3)	0.0038 ± 0.004	0.017 ± 0.005
C302S expressed in M15[pRep4]	0.0005 ± 0.0006	0.017 ± 0.017
C537S expressed in M15[pRep4]	0.0073 ± 0.0017	0.02 ± 0.013

a





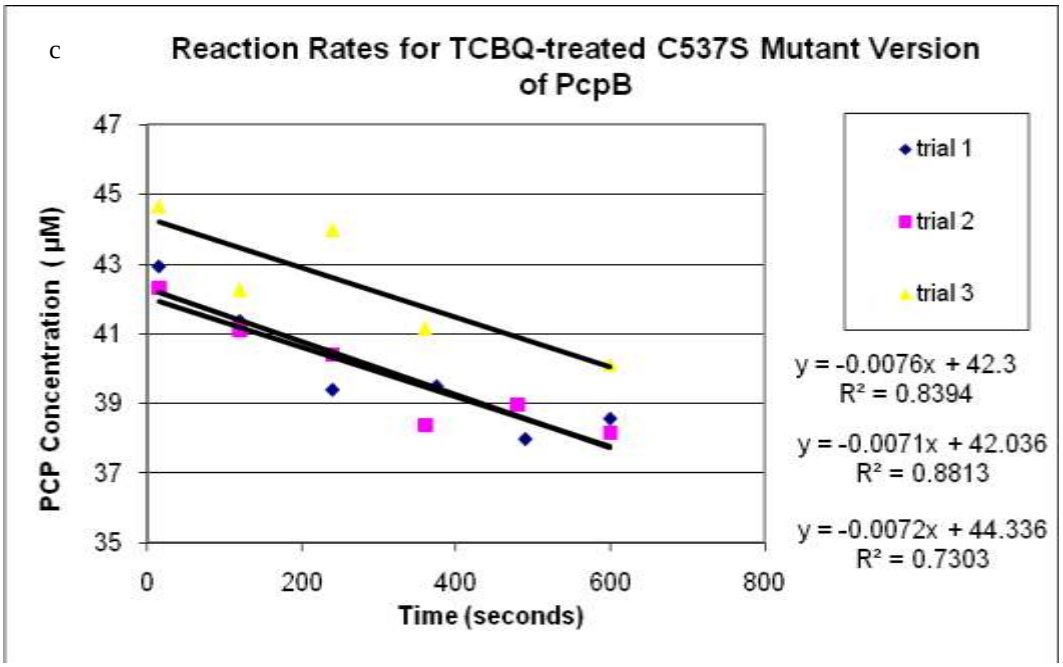


Figure 6. Reaction rates for a) PcpB after treatment with TCBQ, b) C302S mutant version of PcpB after treatment with TCBQ, and c) C537S mutant version of PcpB after treatment with TCBQ. The equations for best-fit lines are in order by trial.

Treating the C302S mutant version of PcpB with 500 µM TCBQ for 60 minutes was sufficient to inactivate it. PCP turnover rate was followed by HPLC for these TCBQ-treated enzymes (n=7 for untreated enzyme, n=2 for TCBQ-treated enzyme) and was also dramatically decreased (Table 2).

After treating the C537S mutant version of PcpB with 500 µM TCBQ for 60 minutes and measuring PCP consumption by HPLC (n=3 for untreated enzyme, n=6 for TCBQ-treated enzyme), this enzyme was shown to get only four-fold inactivated under these conditions (Table 2). This enzyme was least affected by the treatment with TCBQ among the enzymes assayed. This enzyme may show some promise as it does not get inactivated to the same degree as the other versions of PcpB.

Discussion

A cysteine to serine mutant version of PcpB that is functional and does not get inactivated by TCBQ would provide evidence regarding the mechanism of inactivation. If the mutated residue were near the active site (Cys56, Cys182, or Cys302), then this would suggest that the TCBQ adduct prevents PCP from entering the active site or interferes with the catalytic conformational changes. If the mutated residue is far from the active site (Cys40 or Cys537), then this would suggest that the TCBQ adduct interferes with the proper conformations or conformational changes of the enzyme.

It may also be possible that the mechanism of inactivation by TCBQ is not via formation of an adduct to the enzyme but, rather, via oxidation of the corresponding cysteine residue(s). If this is the case, then the mechanism of inactivation is probably due to an inability for the enzyme to undergo conformational changes necessary for catalysis due to the oxidized cysteine(s), This would not suggest that the corresponding oxidized cysteine residue must be reduced in order for the enzyme to bind its substrates. If the corresponding reduced cysteine residue is required to bind its substrates, then the enzyme would not be able to perform catalysis without this cysteine residue even having not been treated with TCBQ.

The results of this study reveal that the C302S, and C537S mutant versions of PcpB are about as functional as the wild type version of the enzyme, indicating that these cysteine residues are not necessary

for the enzyme to function. However, all these mutants get inactivated at least to some extent after treatment with TCBQ, so none of these single cysteine to serine changes is sufficient to prevent this inactivation entirely. Still, the C537S mutant version of PcpB may show some promise towards this goal.

Future Directions

The C40S and C56S mutant versions of PcpB have not yet been assayed. If either of these mutant versions of the enzyme is functional and does not get inactivated by TCBQ, then a version of the enzyme will have been generated that performs better than the wild type version in that the enzyme does not get inactivated by TCBQ. This improved version of the enzyme would be able to continue to catalyze its reaction for theoretically infinite turnovers with no danger of inactivation upon the formation of TCBQ. If either of these mutants fails to function entirely, even without treatment with TCBQ, then this would suggest that the corresponding cysteine that was mutated is necessary for the performance of the enzyme.

More experiments would then be needed to determine the mechanism by which the enzyme fails to function when lacking that corresponding cysteine residue. It may regard binding the substrates or the flavin cofactor, or it may be involved directly in the catalytic mechanism or important for structure or structural changes involved in catalysis.

If all of the cysteine mutant versions of the enzyme are functional, then none of the cysteine residues in the enzyme is necessary for the enzyme to function. If, however, all of the mutant versions get inactivated by TCBQ, then removal of any single cysteine residue is not sufficient to prevent the inactivation. If this is the case, then future experiments should involve double and triple mutants, and perhaps an enzyme with no cysteine residues. Removing all the cysteine residues would hopefully be sufficient to prevent the inactivation. If all single cysteine mutant versions of the enzyme indeed get inactivated by TCBQ, then it is also possible that the mechanism of the inactivation does not involve cysteine residues. To test this, a version of the enzyme with no cysteine residues should be assayed for inactivation. If this version of the enzyme still gets inactivated by TCBQ, then the mechanism of the

inactivation does not involve cysteine residues, and more experiments would need to be done to determine the correct mechanism of inactivation.

Chapter 4: Methods

Site-Directed Mutagenesis and Sequencing of mutant versions of pcpB

Linda Behlen had cloned genes encoding the C40S, C56S, C182S, C302S, and C537S versions of PcpB into the vector pQE30 (Figure 7), but they contained a mutation that encodes a proline at residue 536, where an arginine should be encoded. Quikchange site-directed mutagenesis was used to reverse this mutation so that clones of the genes encoding each mutant gene correctly encoded an arginine at residue 536. The primer 5'-GAG CGA TGC TTG TGC GCT GCG CCT AAG CTT AAT TAG CTG-3' and its reverse complement were used. Each reaction mix was prepared according to the PfuUltra II Fusion HS DNA polymerase protocol (Stratagene, 2006), except that each reaction contained 1.5 µL quick solution, approximately 125 ng of each primer, and 0.2 mM dNTP mix in a final volume of 50 µL. The quick solution was used to help the DNA polymerase with the long template. The Pfu Ultra II fusion DNA polymerase has an antibody in its active site, keeping it from functioning at room temperature so that the reaction mix can be prepared at room temperature. The parental strands that still contained the unwanted mutation, which were methylated, were digested with *Dpn*1. Because the site-directed mutagenesis and Dpn1 digest reactions took place in solutions of high salt, a cleanup of the Dpn1 digest ion products was performed using the Omega E. Z. N. A. Cycle Pure Kit spin protocol (p.6-7 in manual).

Because the plasmids produced by the site-directed mutagenesis were not supercoiled, they were transformed into the *E. coli* strain NEB10β, which was prepared to have high transformation efficiency so that the plasmids were more likely to be taken up by the bacteria. The plasmids were then extracted from the transformants using the E. Z. N. A. Plasmid Miniprep Kit and sequenced by Macrogenusing the pQEPromotor and pQEReverse primers. Sequences obtained were aligned using the EBI ClustalW2 alignment tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to confirm that they had the desired mutation and the correct sequence otherwise. Plasmids containing desires sequences were transformed into the *E. coli* cloning strain TOP10 using the TSS transformation method (Chung, et. al., 1988) and were transformed into the *E. coli* expression strain M15[pRep4] by electroporation.

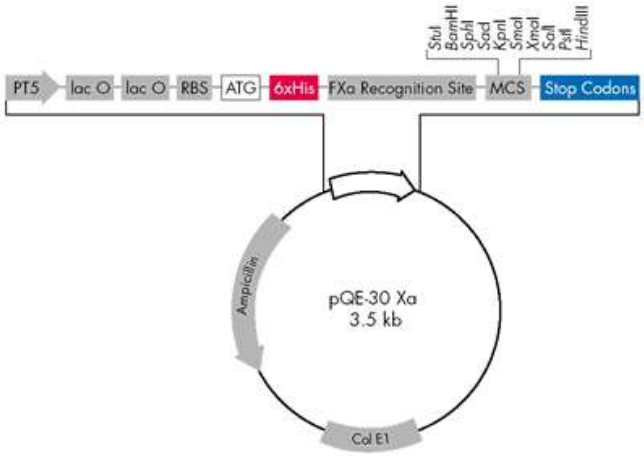


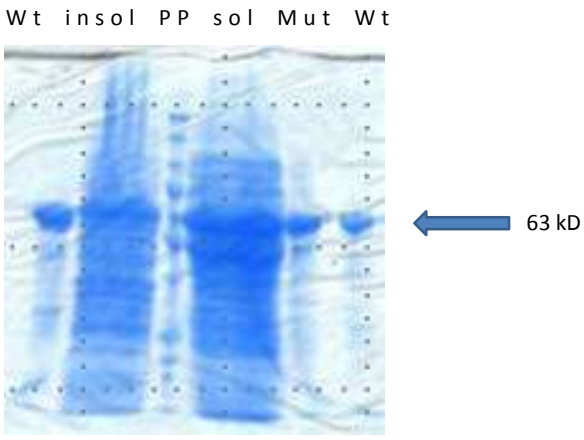
Figure 7. pQE30 plasmid (from Qiagen, 2003, *The QIAexpressionist*), which has a strong lac promoter, a His_6-tag, and an ampicillin resistance gene.

Expression and Purification of Wild type and Mutant Versions of PcpB

Plasmids were transformed into *E. coli* strain M15[pRep4] by electroporation using a Bio-Rad MicroPulser. This expression strain contains the pRep4 plasmid, which encodes a kanamycin resistance gene and strong repressors so that the expression of *pcpB* gene is tightly repressed until induced. Transformants were plated on LB plates containing ampicillin and kanamycin (50 µg/mL each), and one colony of each was inoculated into a small overnight culture in LB containing ampicillin and kanamycin. After overnight growth at 35-37 ^{o}C with shaking, each culture was diluted 100-fold into large cultures of the same medium for expression. Once the cells reach an OD_{600} of about 0.6, expression of PCP hydroxylase was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a concentration of 100 µM, and the cultures were grown at room temperature with shaking. Aliquots were taken at various time points after induction for analysis by SDS-PAGE. Cells were harvested by centrifugation for 10 minutes at 5000 x g at 7-15 ^{o}C. The cells were lysed using a French press three times at a cell pressure of 1135 psi after being resuspended in lysis buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl, 10 mM imidazole, 1 mg/mL lysozyme and 25 U/mL Benzonase Nuclease). The lysates were then subjected to centrifugation at 20,000 x g at 6-13 ^{o}C for 30 minutes. PcpB was purified by Ni-NTA

chromatography, in which the supernatants from the lysates were applied to the column, the column was washed with wash buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl, and 20 mM imidazole), and the protein was eluted with elution buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl, and 250 mM imidazole). The purified proteins were dialyzed against 50 mM phosphate buffer in 50% glycerol. The enzymes were stored at -20^{o}C. The purities of the proteins were determined by SDS-PAGE (Figure 8). The glycerol was diluted before assaying the protein.

a



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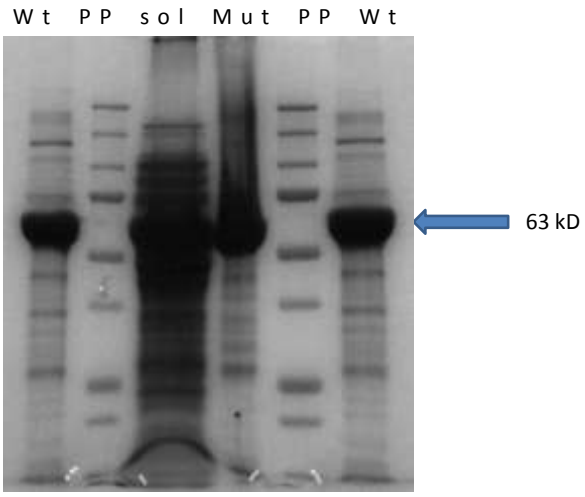

Figure 8. SDS-PAGE gels of a) C302S and b) C537S mutant versions of PcpB expressed in E. coli strain M15[pRep4], showing unpurified lysate and purified protein. The molecular weight of the PcpB monomer is 63 kDa. PP refers to the Bio-Rad Precision Plus Protein Standards.

Determination of Flavin and PcpB Concentration

Purified enzymes were incubated in 7 M urea in 100 mM Tris-HCl, pH 8.0, for a few seconds at room temperature to denature the enzymes and release the flavin. They were diluted to obtain A280 and A446 between 0.1 and 0.5, and the concentration of flavin was calculated using Equation 1.

$$[\text{flavin}] = A_{446}/11.3\ \text{mM}^{-1}\ \text{cm}^{-1} * 1\ \text{cm} \qquad \text{Eq. 1}$$

PcpB and flavin both absorb at 280 nm. For free flavin, $A_{280} = 2*A_{446}$. The extinction coefficient at 280 nm for monomeric apoenzyme PcpB is 68 $\text{mM}^{-1}\ \text{cm}^{-1}$. PcpB concentration was calculated using Equation 2.

$$[PcpB] = (A_{280} - 2*A_{446})/68\ mM^{-1}\ cm^{-1} * 1\ cm \qquad \text{Eq. 2}$$

For PcpB fully loaded with flavin, the ratio of the concentration of flavin to the concentration of monomeric PcpB should be 1:1. The flavin loading for each enzyme was determined by the ratio of the concentration of free flavin to the concentration of PcpB obtained from the A_{280} and A_{446}.

Assay for PCP hydroxylase activity

PCP hydroxylase activity was assayed at room temperature in 100 mM potassium phosphate buffer, pH 7.5, containing 300 μM NADPH, PCP hydroxylase, and 50 μM PCP. The K_M for PCP for PcpB is about 2 μM. The K_M for the mutant versions of the enzyme is probably about the same, so the enzymes were saturated at 300 μM NADPH and 50 μM PCP. PCP stock solution was prepared according to Habeeb (1972). The time courses and enzyme concentrations were adjusted so that 10% of the PCP in the reaction mixture was consumed. NADPH stock solutions (10 mM) were prepared fresh each day. The PCP stock solution was vortexed before addition to the reaction tube. Aliquots from various time points were incubated in a sand bath at 130-150 ºC for three minutes to quench the reaction and centrifuged at 16,100 x g for 5 minutes to remove precipitated PcpB prior to analysis by HPLC. Disappearance of PCP was followed by HPLC on a reverse-phase Zorbaxb-SB C-18 column (150 mm x 4.6 mm; Agilent) using an Agilent 1100 HPLC system and detection at 305 nm. The column was equilibrated with 80% 0.01 M phosphoric acid and 20% acetonitrile. A gradient elution method was used with a 0.01 M phosphoric acid and acetonitrile mixture, from 20 to 45% acetonitrile for the first 7.5 minutes, and 45 to 80% acetonitrile for 2 minutes at a flow rate of 1.5 mL/min. The retention time of PCP was 11.6 minutes. The activity was considered in terms of flavin concentration, not enzyme concentration, because the enzyme without flavin is inactive. Formation of product (TCBQ) cannot be followed because it can be nonenzymatically reduced to TCHQ by NADPH in the reaction mixture, and some may covalently attach to cysteine residues on the enzyme.

Inactivation of Wild Type and Mutant PcpB by TCBQ

Enzymes (5 μM) were treated with 500 μM TCBQ in 100 mM phosphate buffer, pH 7.5 for 60 minutes at room temperature. TCBQ stock solutions (10 mM) were prepared fresh in acetone The reaction mixtures were then diluted three-fold into 100 mM phosphate buffer, pH 7.5. The solutions were filtered through Millipore centrifugal filter units to remove any excess TCBQ. The TCBQ-treated and untreated enzymes were assayed for activity as described above

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